Exhibit G

MOLECULAR BIOLOGY OF THE CELL

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"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at sometime has been, a cell."

Edmund B. Wilson
The Cell in Development and Heredity
3rd edition, 1925, Macmillan, Inc.

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Recombinant DNA Technology[27]

In the early 1970s DNA was the most difficult cellular compound for the biochemist to analyze. Enormously long and chemically monotonous, the nucleotide sequence of the hereditary material could be approached only by indirect means—such as through protein or RNA sequencing or by genetic analysis. Today the situation has entirely changed. From being the hardest macromolecule of the cell to analyze, DNA has become the easiest. It is now possible to excise specific regions of DNA, to obtain them in essentially unlimited quantities, and to determine the sequence of their nucleotides at a rate of several hundred nucleotides a day.

The new **recombinant DNA technology** has provided powerful and novel approaches to understanding the complex mechanisms by which eucaryotic gene expression is regulated, and it has largely superceded conventional methods for determining the amino acid sequence of a protein. Elaborations of the same methods offer great commercial promise for the large-scale economical production of protein hormones and vaccines, available at present only with great labor and cost.

Recombinant DNA technology comprises a mixture of techniques, some new and some borrowed from other fields such as microbial genetics (Table 4–12). The most important ones are (1) specific cleavage of DNA by *restriction nucleases,* (2) *nucleic acid hybridization,* which makes it possible to identify specific sequences of DNA or RNA with great accuracy and sensitivity by their ability to bind a complementary nucleic acid sequence, (3) *DNA cloning,* whereby a specific DNA fragment is integrated into a rapidly replicating genetic element (plasmid or virus) so that it can be amplified in bacteria or yeast cells, and (4) *DNA sequencing* of the nucleotides in a cloned DNA fragment.

Restriction Nucleases Hydrolyze DNA Molecules at Specific Nucleotide Sequences[28]

Many bacteria make enzymes called **restriction nucleases,** which protect them by degrading any invading foreign DNA molecules. Each enzyme recognizes a specific sequence of four to six nucleotides in DNA. The correspond-

Table 4–12 Major Steps in the Development of Recombinant DNA Technology

1869	**Miescher** isolated DNA for the first time.
1944	**Avery** provided evidence that DNA, rather than protein, carries the genetic information during bacterial transformation.
1953	**Watson and Crick** proposed the double-helix model for DNA structure based on x-ray results of **Franklin and Wilkins.**
1961	**Marmur and Doty** discovered DNA renaturation, establishing the specificity and feasibility of nucleic acid hybridization reactions.
1962	**Arber** provided the first evidence for the existence of DNA restriction enzymes, leading to their later purification and use in DNA sequence characterization by **Nathans and H. Smith.**
1966	**Nirenberg, Ochoa,** and **Khorana** elucidated the genetic code.
1967	**Gellert** discovered DNA ligase, the enzyme used to join DNA fragments together.
1972–1973	DNA cloning techniques were developed by the laboratories of **Boyer, Cohen, Berg,** and their colleagues at Stanford University and the University of California at San Francisco.
1975–1977	**Sanger and Barrell** and **Maxam and Gilbert** developed rapid DNA-sequencing methods.

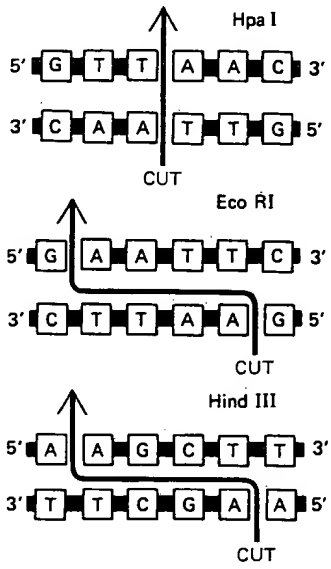

Figure 4–46 The DNA nucleotide sequences recognized by three widely used restriction nucleases. Such sequences are often, as in these examples, six base pairs long and "palindromic"—that is, the nucleotide sequences of the two strands are the same in the recognized region. The two strands of DNA are cut at or near the recognition sequence, often with a staggered cleavage that creates a cohesive end—as for Eco RI and Hind III. Restriction nucleases are obtained from various species of bacteria: Hpa I is from *Hemophilus parainfluenzae*; Eco RI, *Escherichia coli*; and Hind III, *Hemophilus influenzae*.

ing sequences in the genome of the bacterium itself are "camouflaged" by methylation at an A or a C residue, but any foreign DNA molecule that enters the cell is immediately recognized by the nuclease, and both strands of its DNA helix are cut (Figure 4–46). Many restriction nucleases have been purified from different species of bacteria, and more than 100, most of which recognize different nucleotide sequences, are now commercially available.

A particular restriction nuclease will cut any long length of DNA double helix into a series of fragments known as **restriction fragments.** By comparing the sizes of the DNA fragments produced from a particular genetic region after treatment with a combination of different restriction nucleases, a **restriction map** can be constructed that shows the location of each cutting (restriction) site in relation to its neighbors. Since such maps reflect the arrangement of selected nucleotide sequences in the region, a comparison of such maps for two or more related genes will give a rough estimate of the homology between them. For example, the restriction maps, and therefore presumably the nucleotide sequences, of the entire chromosomal regions coding for hemoglobin chains in man, orangutan, and chimpanzee have remained largely unchanged during the 5 to 10 million years since these species first diverged (Figure 4–47).

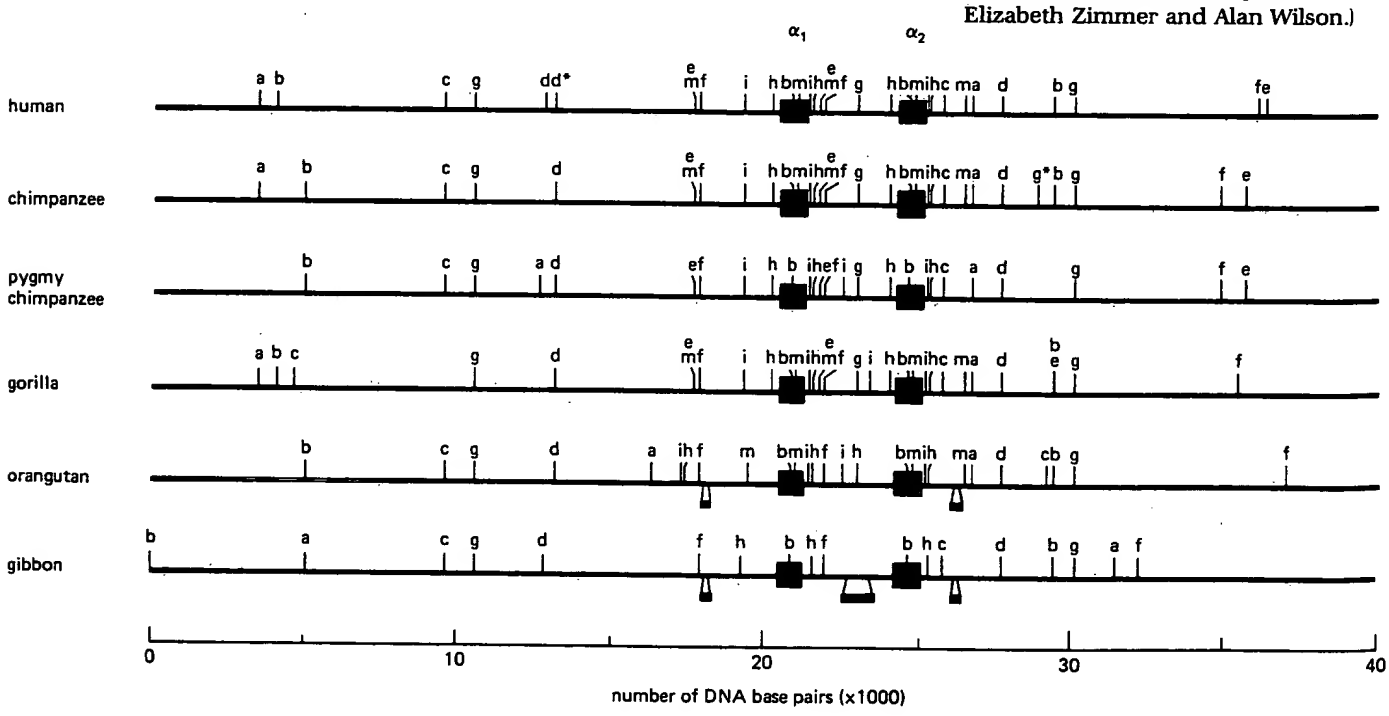

Figure 4–47 Restriction maps of human and various primate DNAs in a cluster of genes coding for hemoglobin. The two squares in each map indicate the positions of the DNA corresponding to the α-globin genes. Each letter stands for a site cut by a different restriction nuclease. The location of each cut was determined by comparing the sizes of the DNA fragments generated by treating the DNAs with the various restriction nucleases, individually and in combinations. (Courtesy of Elizabeth Zimmer and Alan Wilson.)

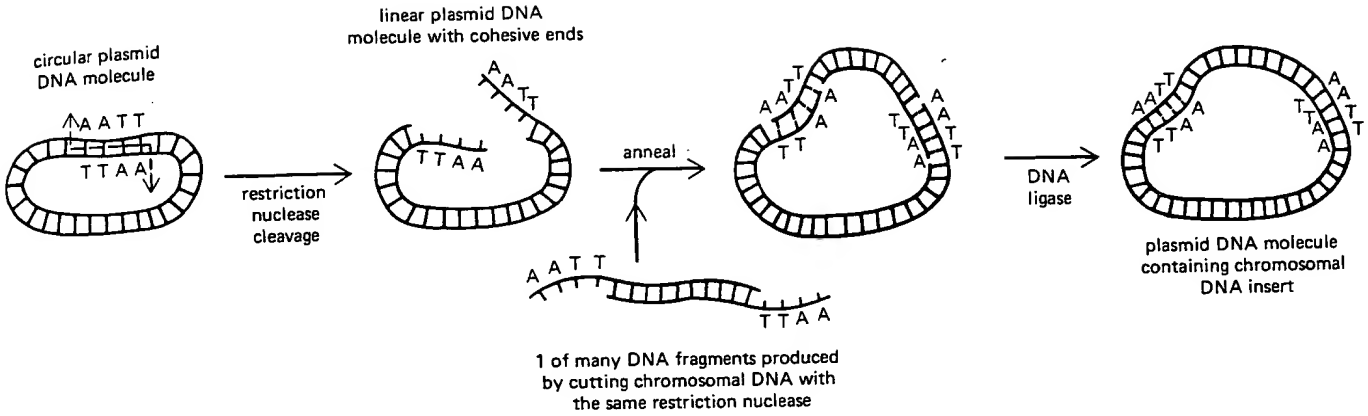

Figure 4–48 The cohesive ends produced by many kinds of restriction nucleases (see Figure 4–46) allow two DNA fragments to be joined by complementary nucleotide base-pair interactions. DNA fragments that are joined in this way can be covalently linked in a reaction catalyzed by the enzyme DNA ligase. In this example, a hybrid plasmid DNA molecule that contains a chromosomal DNA insert is formed.

Many restriction nucleases produce staggered cuts, which leave short, single-stranded ends on both fragments. These are known as *cohesive ends* since they can form complementary base pairs with any other end produced by the same enzyme. A circular DNA molecule that is cut at a single site by this type of restriction nuclease will therefore tend to re-form a circle by the annealing (base-pairing) of its cohesive ends. The cohesive ends generated by restriction enzymes have been very important in recombinant DNA technology because they enable any two DNA fragments to be joined, provided that they were generated with the same restriction nuclease, and thus have complementary cohesive ends. Once the two ends have joined by complementary base-pairing, they can be sealed by an enzyme known as a *DNA ligase*, which forms covalent phosphodiester bonds between the opposing ends of each strand of DNA (Figure 4–48). The combined use of restriction enzymes and DNA ligase has made it possible to graft fragments of any DNA into self-replicating elements.

Selected DNA Sequences Are Produced in Large Amounts by Cloning[29]

Fragments of DNA from any source can be amplified more than a millionfold by inserting them into a *plasmid* or a bacterial virus (*bacteriophage*) and then growing these in bacterial (or yeast) cells—a process called **DNA cloning.** Plasmids are small circular molecules of double-stranded DNA that occur naturally in both bacteria and yeast, where they replicate as independent units as the host cell proliferates. Although they generally account for only a small fraction of the total host cell DNA, they often carry vital genes, such as those that confer resistance to antibiotics. These genes, and the relatively small size of the plasmid DNA, are exploited in recombinant DNA technology.

Because it is so much smaller, plasmid DNA can easily be separated from the DNA of the host cell and purified. For use as *cloning vectors*, such purified plasmid DNA molecules are cut once with a restriction nuclease and then annealed to the DNA fragment that is to be cloned. The hybrid plasmid DNA molecules produced are then reintroduced into bacteria that have been made transiently permeable to macromolecules. Only some of the treated cells will take up a plasmid. They can be selected by the antibiotic resistance conferred on them by the plasmid since they alone will grow in the presence of antibiotic. As these bacteria divide, the plasmid also replicates to produce an enormous number of copies of the original DNA fragment (Figure 4–49). At the end of the period of proliferation, the hybrid plasmid DNA molecules are

purified and the copies of the original DNA fragments excised by a second treatment with the same restriction endonuclease (Figure 4–50).

The DNA to be cloned is often obtained by cleaving the entire genome of a cell with a specific restriction endonuclease. An enormous number of DNA fragments is obtained in this way—anywhere between 10^5 to 10^7 fragments from a mammalian genome, for example. The cloning process, therefore, may produce millions of different bacterial or yeast colonies, each harboring a plasmid with a different inserted genomic DNA sequence. The rare colony whose plasmid contains the genomic DNA region of interest must then be selected and allowed to proliferate to form a large cell population, or *clone*. The selection of the desired colony is often the most difficult part of the cloning procedure. The technique normally used for identifying the colony containing a specific cloned DNA fragment involves the use of radioactive nucleic acid probes complementary to the cloned DNA. We shall now discuss how such probes are commonly made.

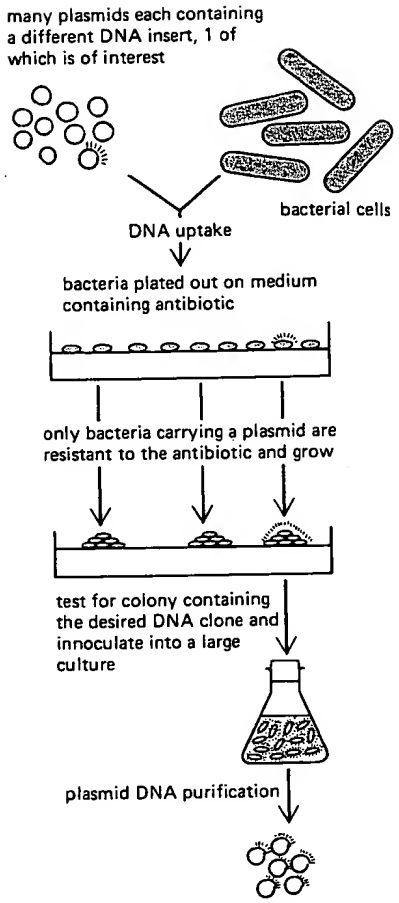

Figure 4–49 Purification and amplification of a specific DNA sequence by DNA cloning in a bacterium. DNA fragments are cloned in yeast cells by a similar procedure.

Copies of Specific mRNA Molecules Can Be Cloned[30,31]

The cloning procedure just described is sometimes called a "shotgun" approach because the entire genomic DNA is cut into an enormous number of fragments that are randomly placed with respect to genes. As a result, some will contain parts of genes and many will contain only *noncoding* DNA and thus no genes at all. An alternative strategy is to begin the cloning process by selecting only those DNA sequences that are transcribed into RNA. This is done by extracting the mRNA (or a purified subfraction of the mRNA) from cells and then making a *DNA copy* (called a **cDNA molecule**) of each mRNA molecule present. This is made possible by an enzyme known as *reverse transcriptase* because, instead of catalyzing the transcription of DNA into RNA, it catalyzes the reverse process of synthesizing a complementary DNA chain on an RNA template. The single-stranded cDNA molecules synthesized by reverse transcriptase can be converted into double-stranded cDNA molecules (by using the enzyme *DNA polymerase*), inserted into plasmids, and cloned (Figure 4–51).

It is possible to construct plasmids in a way that allows the cloned cDNA to direct the synthesis within a cell of large amounts of the particular protein that the cDNA specifies. By means of such "genetic engineering," bacteria or yeast can be induced to make useful proteins, such as human insulin, growth hormone, and interferon, in enormous quantities.

Alternatively, cDNA can be used to identify the rare gene-containing clones produced by the shotgun approach. Here the procedure is to make single-stranded cDNA using radioactive nucleotide precursors. The resulting radiolabeled DNA can be hybridized to the complementary genomic clone in a way that will be described below. Because the cDNA is made from mRNA, it will

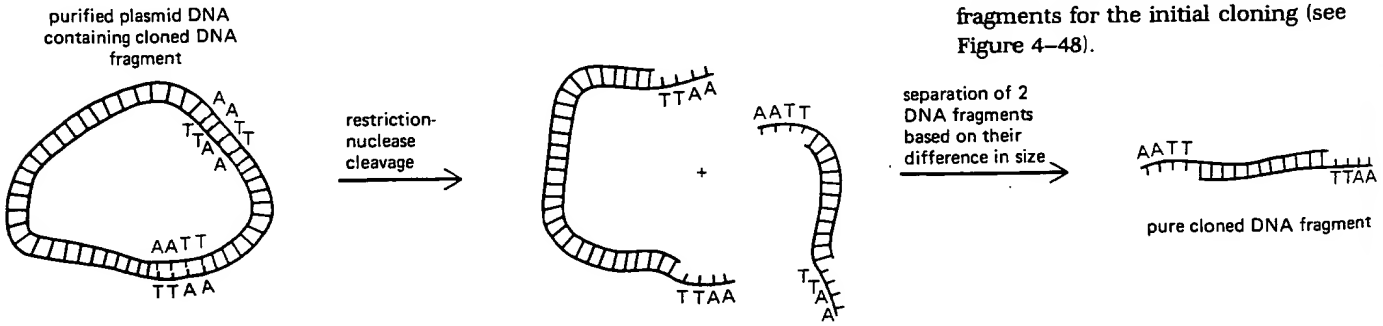

Figure 4–50 Recovery of a cloned DNA fragment from a plasmid containing a recombinant DNA molecule. The fragment is cut out of the plasmid by the same restriction nuclease that created the DNA fragments for the initial cloning (see Figure 4–48).

correspond to genomic DNA that codes for a protein, and its hybridization to the DNA in a clone marks that clone as one containing part of a gene encoding the mRNA molecule.

Cloned DNA Fragments Can Be Rapidly Sequenced[32]

It has recently become possible to determine the nucleotide sequence of cloned DNA fragments simply and quickly. The principle underlying one of these methods is illustrated in Figures 4–52 and 4–53. As a result of this new technology, the complete DNA sequences of more than 100 mammalian genes have already been determined, including those coding for insulin, hemoglobin, interferon, and cytochrome c. At present, the easiest and most accurate way to sequence the amino acids in a protein is by sequencing its gene and then using the genetic code as a dictionary to convert the nucleotide sequence back to a protein sequence. Although there are, in principle, six different reading frames in which any DNA sequence can be read into protein (three on each strand), the correct one is usually recognized as the only one lacking frequent stop codons (see p. 108). The volume of DNA sequence information is already so large ($>10^6$ nucleotides) that computers must be used to store and analyze it.

Nucleic Acid Hybridization Reactions Provide a Sensitive Way of Detecting Specific Nucleotide Sequences[33]

When an aqueous solution of DNA is heated at 100°C or exposed to a very high pH (pH $\geq$ 13), the complementary base pairs that normally hold the two strands of the double helix together are disrupted and the double helix rapidly dissociates into two single strands. This process, called *DNA denaturation*, was for many years thought to be irreversible. However, in 1961 it was discovered that complementary single strands of DNA will readily re-form double helices (a process called **DNA renaturation** or **hybridization**) if they are kept for a prolonged period at 65°C. Similar hybridization reactions will occur be-

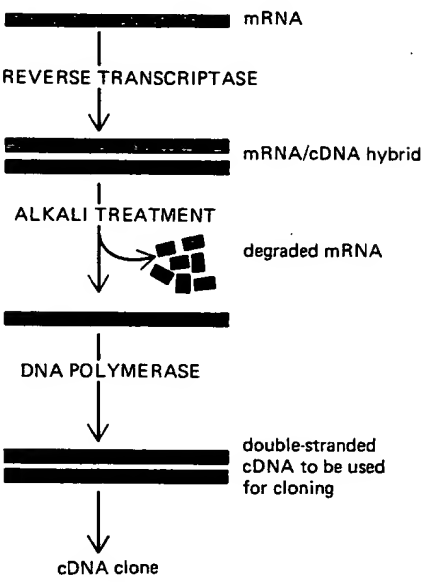

Figure 4–51 A DNA copy (cDNA) of an mRNA molecule is produced by the enzyme reverse transcriptase, a viral enzyme that uses an RNA strand as a template for the synthesis of a complementary DNA strand, thereby forming a DNA/RNA hybrid helix. Treatment of the DNA/RNA hybrid with alkali selectively degrades the RNA strand into nucleotides. The remaining single-stranded cDNA is then copied into double-stranded cDNA by the enzyme DNA polymerase.

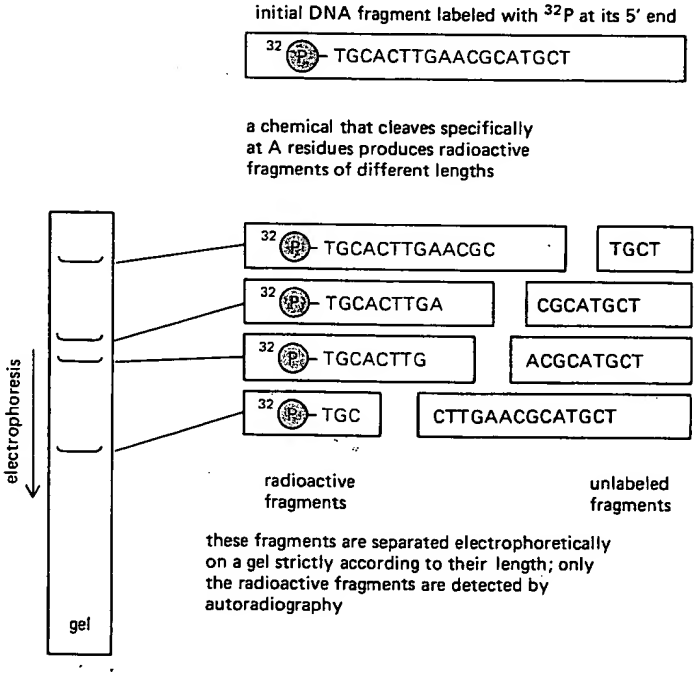

Figure 4–52 The generation of a family of DNA fragments by random cleavage of a DNA chain at a particular type of nucleotide. Each cleavage is produced by a mild chemical treatment that eliminates one nucleotide from the chain while leaving intact most of the nucleotides of the type eliminated. Only the left-hand fragments, possessing a 5′ terminal [^{32}P]phosphate group, are radioactive.

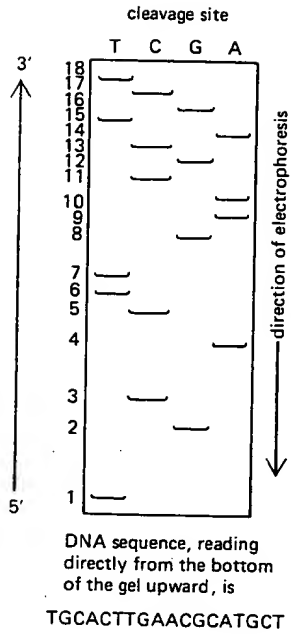

Figure 4–53 Schematic diagram showing one method for sequencing DNA. The type of procedure described in Figure 4–52 is carried out simultaneously on four separate samples of the same DNA using chemicals that cleave DNA specifically at T for the first sample, C for the second, G for the third, and A for the fourth. The resulting fragments are run in parallel lanes of the same gel, giving a pattern from which the DNA sequence is read. The nucleotide closest to the 5′ end of the sequence is determined by looking across the gel at level 1 (at the bottom of the gel) and seeing in which lane a band appears (T). The same procedure is repeated for level 2, then 3, and so on, to obtain the sequence. The method has been idealized here; in actuality the chemical treatments are less specific than shown.

tween any two single-stranded nucleic acid chains (DNA:DNA, RNA:RNA, or RNA:DNA), provided they have a complementary nucleotide sequence.

Because the rate of double-helix formation is limited by the rate at which two complementary nucleic acid chains happen to collide, the concentration of DNA molecules carrying a particular nucleotide sequence can be measured by the rate at which the DNA preparation of interest hybridizes to a radiolabeled cloned DNA probe of complementary sequence. This is such a stringent test that even complementary sequences present in a concentration of one molecule per cell can be detected (Figure 4–54). From such measurements it can be determined how many copies of the DNA sequence contained in the cloned probe are present in the DNA of a cell. While most sequences turn out to be present in only one or a few copies per haploid genome, others are present in hundreds of thousands of copies—the so-called *repeated DNA sequences.*

Alternatively, hybridization studies can be carried out with RNA isolated from cells to determine whether the DNA sequence that has been cloned is one of those transcribed into RNA and, if so, how many copies of the RNA are made per cell and in which types of cells and tissues. Somewhat more elaborate procedures identify the exact region of the cloned probe that hybridizes with cellular RNA molecules and thereby define the start and stop sites for RNA transcription (Figure 4–55); the regions that are cut out of the RNA transcripts during *RNA processing* (the intron sequences) are also identified in this way.

Radioactive cloned DNA probes are widely used to localize specific nucleic acid sequences in mixtures of DNA restriction fragments fractionated by gel electrophoresis. A replica of the gel is made by transferring all of the fractionated DNA fragments to a sheet of nitrocellulose paper either by diffusion or electrophoresis, a process called *blotting.* The locations of the fragments that hybridize to the radioactive DNA probe are then identified by autoradiography (Figure 4–56). In a similar way, nitrocellulose paper replicas can be made of crowded colonies of bacteria growing on an agar surface, so that hybridization of the paper with a specific radioactive probe can be used to identify the few cells carrying a newly cloned specific DNA fragment.

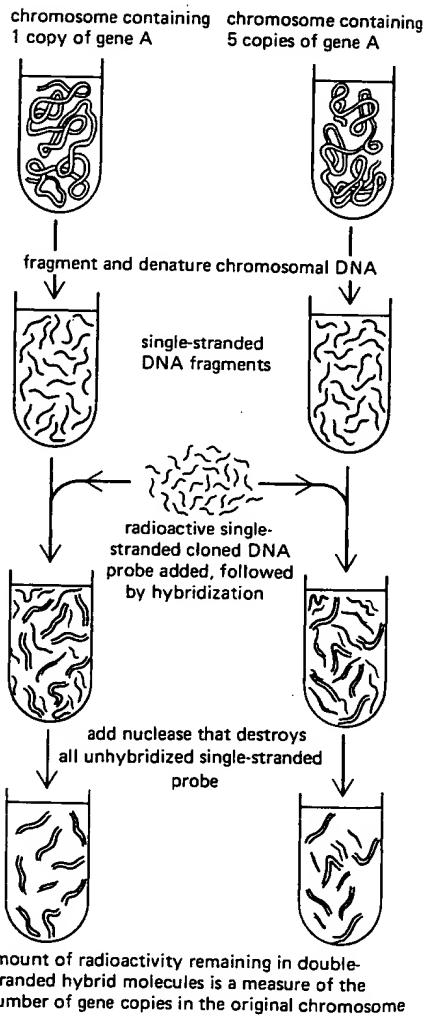

Figure 4–54 The measurement of the number of copies of a specific gene in a sample of DNA by means of DNA hybridization. The radioactive single-stranded DNA fragment used in such experiments is commonly referred to as a *DNA probe;* the chromosomal DNA is not radioactively labeled here.

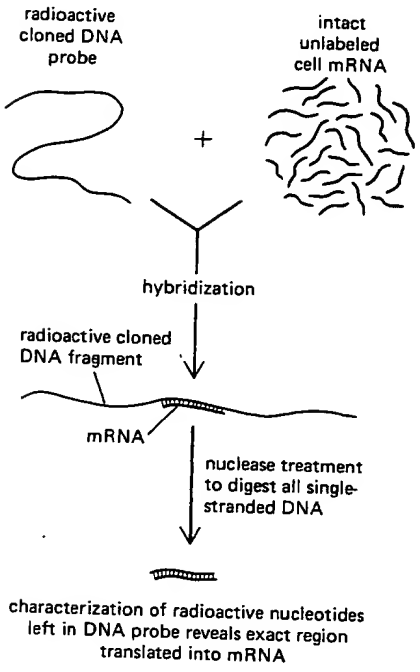

Figure 4–55 The use of nucleic acid hybridization to determine the region of a cloned DNA fragment that is transcribed into mRNA. The existence of intervening sequences (introns) in eucaryotic genes was discovered by this type of procedure.

In Situ Hybridization Techniques Are Used to Localize Specific Nucleic Acid Sequences in Chromosomes and Cells[34]

Nucleic acids, no less than other macromolecules, occupy precise positions within cells and tissues, and a great deal of potential information is lost when these molecules are extracted from cells by homogenization. For this reason, techniques have been developed in which nucleic acid probes are used in much the same way as labeled antibodies to localize specific nucleic acid sequences *in situ*, either in chromosomes or particular types of cells. In the original *in situ* hybridizations, highly radioactive nucleic acid probes were hybridized to squashed, fixed chromosomes that had been exposed briefly to a very high pH in order to disrupt their DNA base pairs. After extensive washing, the chromosomal regions that bound the radioactive probe were visualized by autoradiography (Figure 4–57). Recently, the spatial resolution of this technique has been improved by the development of special methods for labeling the nucleic acid probes with fluorescent dyes.

Similar *in situ* hybridization methods have been useful for detecting the presence of particular growing RNA transcripts on unusually large "lampbrush chromosomes"; here the chromosomes are not exposed to a high pH, so the chromosomal DNA itself remains double-stranded and thus cannot bind the probe. Comparable methods can be used on fixed tissue sections to determine which cells in a complex tissue contain cytoplasmic RNA molecules complementary to a particular DNA probe.

Recombinant DNA Techniques Allow Even the Minor Proteins of a Cell to Be Studied[31,35]

Until very recently, the only proteins that could be readily studied were relatively abundant components of the cell. Starting with several hundred grams of cells, a major protein—one that constitutes 1% or more of the total cellular protein—can be purified by a series of simple chromatographic and electro-

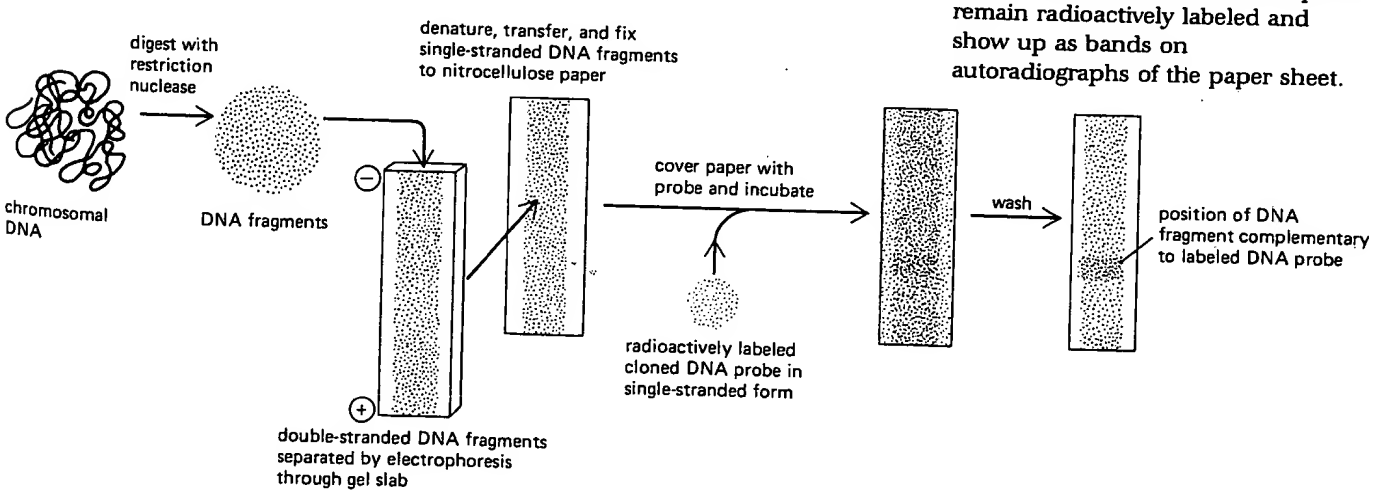

Figure 4–56 After a DNA sample has been cleaved by a restriction nuclease and then separated by electrophoresis, the many different DNA fragments present are transferred to nitrocellulose paper by blotting and then are exposed to a radioactive DNA probe for a prolonged period under annealing conditions. The sheet is washed extensively so that only those DNA fragments that hybridize to the probe remain radioactively labeled and show up as bands on autoradiographs of the paper sheet.

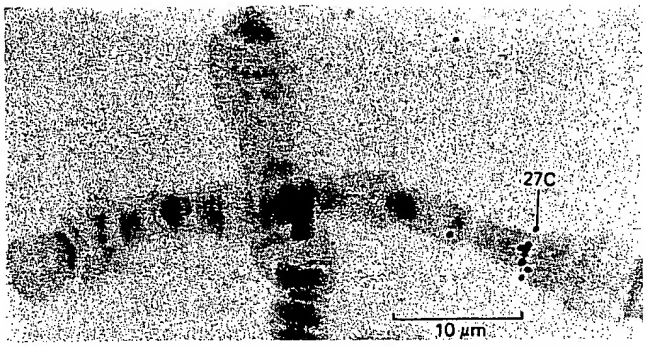

Figure 4–57 Localization of a *Drosophila* gene by *in situ* hybridization of a radioactive cloned DNA probe with *Drosophila* polytene chromosomes. The cluster of darkened silver grains detected in this autoradiograph is located at chromosome map position 27C, as indicated. Parts of two of the four giant chromosomes present in each larval salivary gland cell are shown. (Courtesy of Steven Henikoff.)

phoretic procedures to yield perhaps 0.1 g (100 mg) of pure protein. This quantity of protein is sufficient for conventional amino acid sequencing, detailed analysis of its biological or enzymatic activity (if any), and the production of antibodies, which can then be used to localize the protein in the cell. Moreover, if suitable crystals can be grown, the three-dimensional structure of the protein can be analyzed by x-ray diffraction crystallography. In this way, the structure and function of many abundant proteins have been determined, including hemoglobin, trypsin, immunoglobulin, and lysozyme.

The vast majority of the thousands of different proteins in a eucaryotic cell, including many of the most interesting ones, are present in only very small amounts. For most of them it is extremely difficult, if not impossible, to obtain more than a few micrograms of pure material. In principle, however, recombinant DNA technology has now made essentially any protein in the cell, including the minor ones, accessible to the same structural and functional studies that were previously possible only for a rare few. A summary of the steps that make this possible is given in Table 4–13.

Mutant Genes Can Now Be Made to Order[36]

Suppose one isolates a new protein from a cell extract and clones its gene by the "shotgun" technique described above. How can one discover what the protein does in the cell? The problem is surprisingly difficult since neither the three-dimensional structure of the protein nor the complete nucleotide sequence of its gene identifies the protein's function. And many proteins, such as structural components of the cell or proteins that are normally part of a large multienzyme complex, have no obvious activity when they are separated from the other components of the functional unit.

One approach already discussed is to inactivate the particular protein by means of a specific antibody. When combined with the technique of microinjection, this provides a powerful probe to test protein function. However, some antigenic sites on proteins will be inaccessible to antibody molecules even if the antibodies are injected into the cytoplasm. Furthermore, many antibodies bind to protein molecules without inactivating them.

Genetic approaches provide an elegant solution to this problem. Mutants that lack a particular protein or, more usefully, synthesize a temperature-

Table 4–13 Steps in the Purification of Large Amounts of a Minor Protein of the Cell Using Recombinant DNA Technology

1. Fractionate the cell extract by a series of conventional chromatographic procedures until the protein of interest is sufficiently enriched that a microgram can be obtained in pure form by cutting it out of a gel following high-resolution gel electrophoresis.
2. Analyze the denatured protein on a microsequenator to determine the sequence of the first 30 amino acids at its amino terminus.
3. Use the genetic code to predict the nucleotide sequences in mRNA corresponding to the above amino acid sequence. Using rapid chemical methods, synthesize a set of short DNA fragments, 15 to 20 nucleotides long, 1 of which will form complementary base pairs with part of the mRNA sequence. (There will be some ambiguity here since several different codons code for the same amino acid—p. 108).
4. Hybridize these short DNA fragments to total cellular mRNA and use them to direct reverse transcriptase to the mRNA molecules with complementary sequences. The reverse transcriptase then copies these complementary mRNA molecules to produce long cDNA molecules (Figure 4–51).
5. Produce large amounts of DNA containing the sequence of each of these cDNA molecules by cloning (Figures 4–49 and 4–50).
6. Hybridize DNA prepared from each cDNA clone to total cellular mRNA and thereby select and purify mRNA molecules that are complementary in sequence to each cloned cDNA sequence.
7. Translate each mRNA preparation obtained into protein by cell-free protein synthesis in order to determine which one codes for the desired protein.
8. Sequence the appropriate cDNA (Figure 4–53) and use the genetic code to determine the protein's complete amino acid sequence and where the coding sequence for the protein begins and ends.
9. Insert the cloned cDNA sequence into a specially engineered plasmid DNA vector containing inserted transcription and translation start signals. Use bacterial or yeast cells containing this new plasmid clone as the starting material for the isolation of large amounts (100 mg or more) of the purified protein.

sensitive version of the protein that is inactivated by a small increase (or decrease) in temperature may quickly reveal the function of the normal molecule. While this approach has been immensely useful, for example, in elucidating the principal metabolic pathways of bacteria, it has been mainly restricted to very rapidly replicating organisms—such as bacteria, yeast, nematode worms, and fruit flies—where very large numbers of mutants can be quickly isolated and then screened for a particular defect of interest.

In principle, the genetic approach can now be made more generally applicable by creating specific "mutations" outside the cell. With recently developed methods, a copy of an isolated cloned gene can be altered slightly by biochemical means and then put back into a cell, which now synthesizes an altered protein. In bacterial and yeast cells, this mutant gene will recombine with the normal gene often enough to make it possible to select for cells in which the mutant gene has replaced the single copy of the normal gene. In this way, cells carrying a specific protein in mutant form are made to order and the phenotype of a cell that lacks the normal gene thereby determined. Similar methods are not yet available for inserting a cloned mutant gene back into mammalian cells in place of the normal gene, but with the extraordinarily rapid rate of progress in recombinant DNA technology, it would not be surprising if this soon becomes possible.

Summary

Recombinant DNA technology has revolutionized the study of the cell. Any region of the cell's DNA can now be excised with restriction nucleases and produced in virtually unlimited quantities by DNA cloning and then sequenced at rates of hundreds of nucleotides a day. As a result, many genes and noncoding regions of the eucaryotic genome have already been sequenced.

By using nucleic acid hybridization methods, mRNA molecules corresponding to cloned DNA molecules can be detected, isolated, and translated into protein in cell-free systems. Furthermore, it is possible, in principle, to work backward from a protein to the gene that encodes it: by using a short stretch of amino acid sequence from the protein, specific DNA probes can be synthesized that will hybridize with the mRNA and DNA encoding the protein.

The practical consequences of recombinant DNA technology are far-reaching. Bacteria or yeast can be engineered to make a mammalian protein in virtually unlimited quantities, making it possible to analyze the structure and function of the protein or to use the protein as a vaccine or drug for medical purposes.

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